

BACTERIAL ECOLOGY OF STRIP MINE AREAS AND ITS RELATIONSHIP TO THE PRODUCTION OF ACIDIC MINE DRAINAGE^{1, 2}

PATRICK R. DUGAN

Department of Microbiology, The Ohio State University, Columbus 43210

ABSTRACT

DUGAN, PATRICK R. Bacterial ecology of strip mine areas and its relationship to the production of acidic mine drainage. Ohio J. Sci. 75(6): 266, 1975.

The activity of acidophilic bacteria as agents involved in the production of sulfuric acid from iron pyrite (FeS_2) found in association with coal mine refuse or spoils was reviewed. Data was presented which demonstrated the inhibitory effect of anionic detergents and certain organic acids on the growth and metabolism of the acidophilic thiobacilli. The influence of acidic mine drainage on the microflora of non-acid polluted streams was considered. Also discussed were the heterotrophic microbes which are indigenous to acid (pH 3.0) streams and acid coal refuse, with a section devoted to the potential for sulfate reducing bacteria as agents for removal of sulfuric acid from the streams.

It has been estimated that 35% of the world's energy is presently consumed in the United States; of which 22% is derived from coal (Osborn, 1974). This is the equivalent of 600 to 700 million tons per year. As of 1971, 40% of the U.S. coal was being extracted via stripping operations. Two million acres of land have already been strip mined in order to achieve this level of production, however, only 0.3% of the stripped land has been reclaimed (Liptak, 1974). The incentive for stripping coal is economic, with the cost of strip mined coal estimated to be \$1.25 per ton less than deep mined coal. In Ohio, a considerably higher proportion of coal is strip mined

(70%) as compared to deep mined (30%). This was equivalent to about 50 million tons of strip mined coal in Ohio for the year 1972 (Land Reborn, 1974).

The three general types of mining are schematically illustrated in figures 1A, 1B and 1C. Each shows a seam of coal located under different terrains. If there is a sufficient depth of rock and soil (*overburden*) overlying the coal, it was more feasible to sink a vertical shaft to reach the coal (fig. 1A). Overburden usually consists of sandstone, limestone clay and/or shale which may contain other sedimentary deposits such as pyritic minerals. Pyritic minerals and shale are often found in intimate contact with the coal or sandwiched between seams of coal. Some of the shale, low grade, non-marketable coal, pyrite and other non-marketable minerals are extracted with the coal and left on the surface when the coal is cleaned and hauled to market. These piles of minerals are known as *refuse piles* and are sometimes referred to as "gob piles". Several kinds of pyritic minerals are found in nature, but iron pyrite (FeS_2), also known as fool's gold, is the one most commonly encountered in association with coal.

When the terrain is such that the coal seam *outcropped* along the side of a slope (fig. 1B), it was more expedient to *auger* into the hillside. This is known either as an *auger hole* or *drift mine*. The refuse pile is not shown in figure 1B but would be present in the area. There is virtually no way to segregate pyrite from other minerals and low grade coal in refuse originating from either deep or drift mines.

When a coal seam is sufficiently near the surface, a large shovel or drag line can be used to scoop away the overburden and expose the seam of coal as illustrated in figure 1C. Shovels with a bucket capacity of 135 cubic yards and drag-

¹Manuscript received July 22, 1975, revised November 3, 1975 (#75-48).

²Presented as part of a symposium, Biological Implications of Strip Mining, held at Battelle Memorial Institute, Columbus, Ohio, on November 15, 1974.

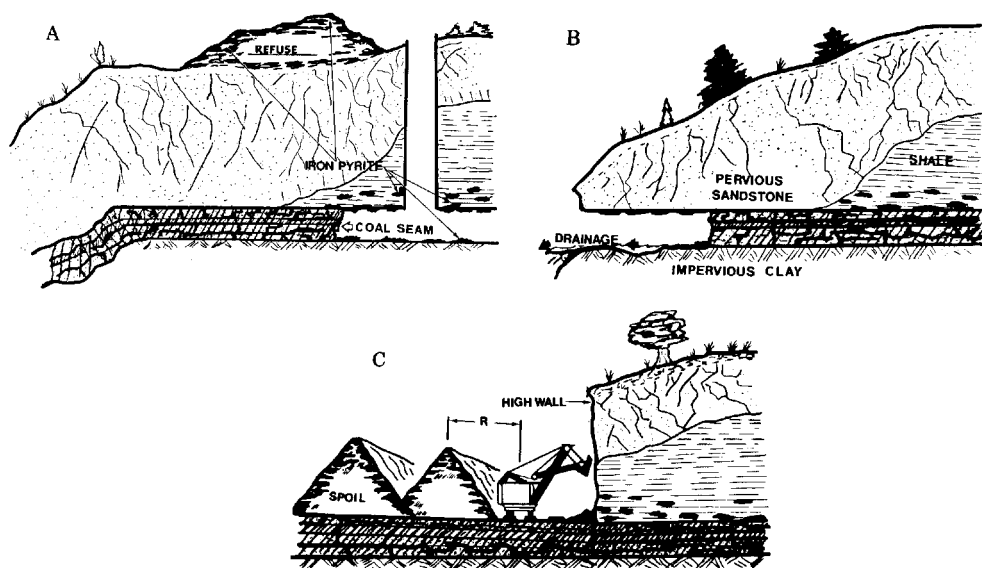
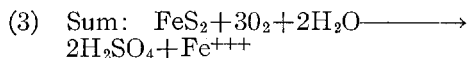
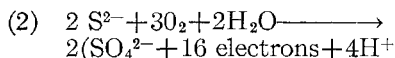
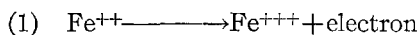


FIGURE 1. Schematic drawing of three general types of coal mining and association of coal with pyrite. (A) deep mining with shaft and refuse pile, (B) auger or drift mine illustrating flow of water drainage, (C) strip mining illustrating rows of overburden spoil banks, high wall.

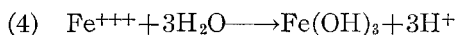
lines with 220 cubic yards bucket capacity are presently used in Ohio. Once the overburden has been stripped away, a smaller shovel follows behind and scoops up the coal seam and loads it into trucks, railroad gondolas, etc. Each strip of overburden removed is successively piled behind the shovel as the shovel "chews" its way along the contour of the hillside. Consequently, overburden which may contain pyrite is piled in a series of wind row banks separated by a distance equal to the radius of the shovel fulcrum. After stripping in an area is terminated, a "highwall" remains as the result of the final cut into the hillside. It is often possible to segregate pyrite from other minerals in overburden during a stripping operation and bury it deep in the spoil bank. The term *spoil* is used in conjunction with stripped overburden, in contrast to the refuse or "gob" from subsurface mines.

One of the environmental concerns associated with coal mining results from the exposure of pyritic minerals to air (oxygen) and moisture. Exposure of the chemically reduced substance FeS_2 to oxygen and water results in oxidation of

the FeS_2 to H_2SO_4 by a complex series of chemical reactions which are summarized in the following equations:



The oxidized iron (Fe^{3+}) formed, subsequently reacts with water to produce ferric hydroxide and more acid according to the following equation:



Everyone who has seen drainage from mining regions where pyrite has been exposed will recognize the yellowish brown or reddish brown precipitate, often called "yellowboy", that forms on streambeds. This is the $\text{Fe}(\text{OH})_3$ formed in equation (4) and is equivalent to rusted or oxidized iron. Ferric hydroxide also reacts with sulfuric acid to form ferric hydroxy sulfate complexes according to equation (5), hence the difference in color and composition of precipitates depend-

ing upon environmental conditions. $\text{Fe}(\text{OH})_2^+$ may also be present in acid solution.

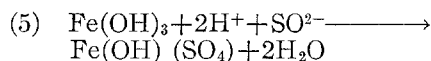


Figure 2 is a schematic illustration of the hydrologic cycle in a refuse pile. Moisture can penetrate all levels of the porous pile and moisture retention, as well as moisture content, depends upon the

The rate and amount of acid production within the piles is determined by many factors such as: the amount of pyrite, particle size of pyrite, presence of microorganisms which oxidize the pyrite, depth of oxygen penetration, moisture content of the pile, and temperature range of the pile, and other factors which we presently do not understand.

Acid appears to be produced at a relatively consistent rate in a given spoil

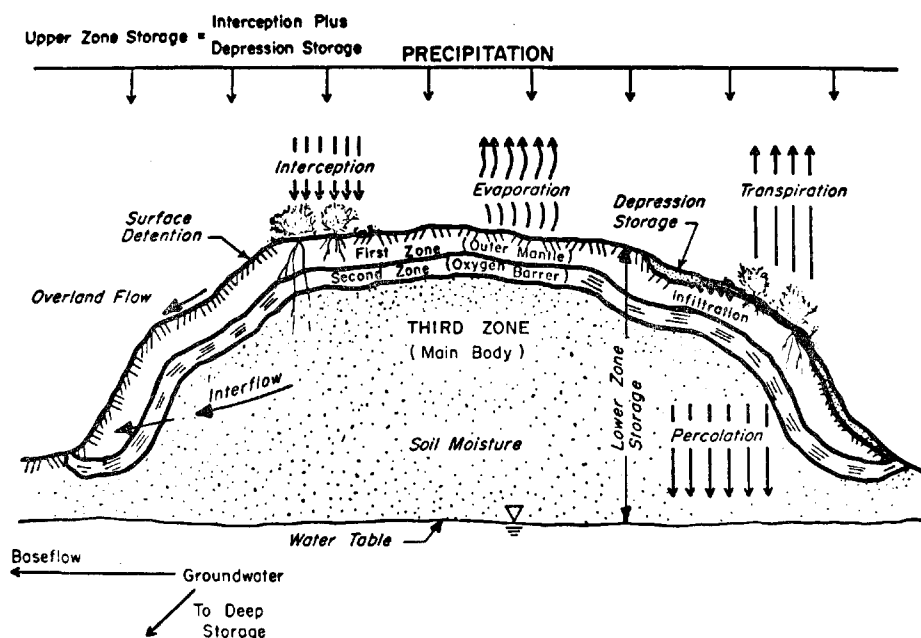


FIGURE 2. Schematic illustration of a cross-section of a refuse pile and its hydrology. (Courtesy of Dr. V. Ricca).

composition of the pile (e.g. the clay, coal, pyrite and sandstone content). Oxygen ordinarily does not penetrate into the pile depth beyond about 8 to 12 inches and is limited by a zone defined as the oxygen barrier which results from compaction of fine sediments (Truax Trayer Coal, 1971). It is the propensity of refuse piles to produce sulfuric acid (H_2SO_4) via the oxidation of iron pyrite (or other sulfur containing minerals) (equation 3) which is the primary basis of our biological concern with strip mining. The sulfuric acid leaches or is flushed out of the pile at a rate determined by local precipitation and ground water flow (Good *et al.*, 1970; Truax Trayer, 1971).

pile and it can be washed out in a surge during a rainfall resulting in a rapid slug-dose of acid entering a drainage or receiving stream. Continued high water flow due to rainfall will subsequently tend to dilute the acid concentration of the drainage (Dugan and Randles, 1968; Shumate *et al.*, 1971). Tests with controlled sprinkling of refuse piles indicated that about 25% of the water applied was retained in the refuse pile while 75% flushed through almost immediately (Good *et al.*, 1970; Vimmerstedt *et al.*, 1973). Acid loads averaging 305 lbs per acre per day have been shown to be flushed out of refuse piles, and slug loads of as high as 6,600 lbs/acre/day have

been recorded. The drainage from such piles may contain as much as: 60,000 mg SO_4 /l (6%), 15,000 mg Fe/l (1.5%), and 60,000 mg net acidity/l (Truax Trayer, 1971).

It has been calculated that 10^5 square miles of land across the 11 states that make up the Appalachia coal mining region produce acidic mine drainage, which pollutes 10,500 miles of streams (Acid Mine Drainage in Appalachia, 1969). It is estimated that more than 370,000 acres of strip mined land will require some form of reclamation in Ohio. Of this total, 180,000 acres are inactive or abandoned acres that will require a major reclamation effort at an estimated cost of \$290 million (in 1973) dollars. Abandoned mines discharge over 1 million lbs of acid per day into Ohio streams. The acid in the streams is highly corrosive to bridges, dams and other structures as well as to plumbing. The toxicity and hardness of the water restricts its use for irrigational and livestock watering purposes as well as for recreational purposes. In general, water contaminated by acid mine drainage seriously retards virtually all beneficial water uses at tremendous economic loss (Land Reborn, 1974). The problem will be intensified by the projected need for a 50% increase in coal production by 1980.

MICROBIAL ECOLOGY

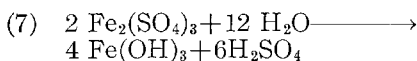
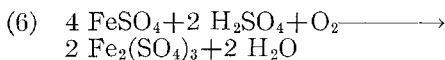
The involvement of microorganisms with acidic mine drainage occurs in at least 4 ways:

- (A) the increased production of acid via the metabolic activity of the acidophilic *Thiobacillus* bacteria,
- (B) the inhibitory influence of sulfuric acid on the organisms normally present in receiving streams,
- (C) growth of acid tolerant microbes which will aid in recovery of acid contaminated streams,
- (D) the ability of sulfate-reducing bacteria to convert sulfate (e.g. H_2SO_4) back to sulfide which can be precipitated as iron sulfide (FeS).

ACID PRODUCTION BY BACTERIA

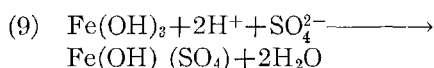
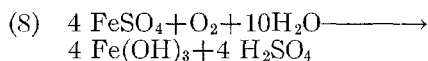
Acidophilic bacteria of the *Thiobacillus-Ferrobacillus* group (i.e. *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* (syn. *Ferrobacillus ferrooxidans*)) have long been recognized as being associated with pyrite oxidation and acid production in coal mine spoils and can be readily isolated from acid mine drainage water (Duncan *et al.*, 1967; Lorenz, 1967; Silverman, 1964). These are bacteria which derive their energy from the oxidation of reduced iron (Fe^{2+}) and sulfur compounds, (both of which are present in iron pyrite) and derive their cellular carbon from CO_2 . The bacteria grow optimally in the pH range of 2.8 to 3.5. Maintenance of an adequate supply of Fe^{2+} as an energy source in the absence of high concentrations of organic material requires an environmental pH less than 4.0 because of the rapid auto-oxidation of Fe^{2+} in the presence of O_2 above pH 4.0.

Prior to reports by Colmer and Hinkle in 1947 of the involvement of *T. ferrooxidans* in the oxidation of pyritic minerals (Colmer *et al.*, 1949), Carpenter and Henderson (1933) suggested the following reactions (6 and 7) to explain the transformation of FeSO_4 produced from pyrite:



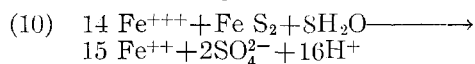
In accordance with equation (6), *T. ferrooxidans* growing on Fe^{2+} consumes oxygen in the ratio of one mole O_2 per four moles of Fe^{2+} oxidized to Fe^{3+} (Lees *et al.*, 1969; Silverman, 1967). The energy being derived from the four electrons liberated from Fe^{2+} . The electrons must be accompanied by protons, for which there is no biological source, since Fe^{3+} is not known to be deposited in the cell, and this may possibly explain the dependence of the organism on an acid environment. Equation (7) is a non-biological reaction that furnishes an environmental supply of H^+ . Ferric hydroxide will react to form hydroxy sulfate complexes (equation 9) which have buffering capacity and can alter equa-

tion (7) and (8) so that less H_2O enters the reactions and less H_2SO_4 is produced. Equation (6) does not consider the proton requirement for the metabolic reduction of CO_2 which is also a requirement of the bacteria.



The pH optima of all enzymes purified from *T. ferrooxidans* are considerably higher (i.e. > pH 5.0) (Blaylock and Nason, 1963; Din *et al.*, 1967; Howard, 1970; Silver, 1968a, 1968b; Vestal, 1971) than the environmental pH (i.e. > 4.0), with the possible exception of the crude preparation of the cell envelope associated iron oxidase reported by Bodo and Lundgren (1974) (optimum pH 2.5 to 3.5). This suggests that the cell envelope of obligate acidophiles selectively excludes high concentrations of H^+ from entering the cell; a conclusion which has been supported by a more direct assessment of the internal pH of *T. ferrooxidans* cells when the external pH is considerably more acid (Dewey, 1966). Beck (1960) suggested that *T. ferrooxidans* has a passive H^+ barrier since resting cells do not respire and are capable of surviving long periods of storage under acidic conditions.

Although iron pyrite will be oxidized chemically in the absence of bacteria, ultimately to $\text{Fe}(\text{OH})_3$ and H_2SO_4 , the bacteria catalyze the reaction and increase the rate of oxidation up to one million times the chemical rate (Singer, 1970). It has been shown that the iron oxidizing bacteria are more active than the sulfur oxidizing bacteria with respect to rates of pyrite oxidation (Leathan *et al.*, 1953). This led to speculation that the primary role of bacteria in pyrite oxidation was the production of ferric ions (equation 1), and that the ferric ions thus produced oxidized more pyrite with concomitant regeneration of ferrous ions, according to equation (10). The recycled iron could again be oxidized by the bacteria and the cycle would continue (Silverman, 1967; Singer and Stumm, 1970).



Singer and Stumm (1970) have concluded that under acidic conditions below pH 4.0, the rate of pyrite oxidation by ferric ion is considerably greater than the rate of ferrous ion oxidation in the absence of bacteria. Therefore, the bacteria must catalyze the oxidation of ferrous to ferric ion in order to supply the Fe^{+3} to oxidize the pyrite. They concluded that the bacterially catalyzed reaction controls the rate of pyrite oxidation under acidic conditions. Lau *et al.* (1970) also came to essentially the same conclusion in that the bacteria were essential to the maintenance of the high ferric to ferrous ion ratio, in solution, which is necessary to chemically oxidize pyrite. The mechanism of sulfur oxidation by *T. thiooxidans* would be somewhat different in that sulfur is essentially insoluble, therefore requiring direct contact of bacterium to substrate (Vogler and Umbreit, 1941).

It should be mentioned that Walsh and Mitchell (1972) have been concerned with the ecological question of how freshly mined spoils initially achieve sufficient acidity to be optimal for the acidophilic *Thiobacilli*. These investigators have isolated a different microorganism, *Metallogenium*, which also produces some acid via the oxidation of Fe^{2+} to Fe^{3+} when accompanied by subsequent hydrolysis according to equation 4. The acid produced creates an environment suitable for the succession of the *Thiobacilli*. Although this may be an ecological explanation, it need not be a prerequisite to establishing *T. ferrooxidans* growth since chemical oxidation of pyrite would accomplish a lowering of pH to about 4.0 over a longer time span.

Much of our knowledge of the microbial oxidation of pyrite has been based upon laboratory investigations of the activity of isolated or enriched cultures of bacteria and relatively little data is available on investigations in the field. One of the most convincing field studies showing the activity of bacteria in refuse piles was carried out by Belly and Brock (1974). These investigators showed a strong correlation between uptake of $^{14}\text{CO}_2$ and most probable numbers (MPN) of iron oxidizing bacteria, but not with the acid tolerant heterotrophic micro-

organisms which were also present in the refuse. They reported maximal $^{14}\text{CO}_2$ uptake in coal refuse 2 to 3 years old with only slight incorporation in fresh material or material 40 years old. Maximal uptake was always found in samples taken from the surface above 8 to 10 cm depth, at temperatures between 20° and 30°C and at moisture content of between 23 and 35%, all of which agree with data previously published on the basis of laboratory investigations (Lau *et al.*, 1970; Truax-Trayer, 1971).

Although actual bacterial catalysis of pyrite oxidation may occur near the surface of refuse piles, the high content of Fe^{+3} dissolved in acid may leach through the spoil banks (See fig. 2) and catalyze the reaction, shown in equation (10), deep within the pile. Oxidation would take place at a slower rate within the pile resulting in a drainage relatively high in Fe^{+2} which could then be re-oxidized by bacteria in drainage streams or wherever oxygen and CO_2 were available.

INHIBITION OF BACTERIAL ACID PRODUCTION

It has become increasingly apparent that bacteria play a major role in the production of acidic mine drainage and it has been argued that the most sensible approach to the control of mine drainage pollution would be to prevent its formation at the source. One specific suggestion was to selectively inhibit the acidophilic *Thiobacilli* in refuse and spoil piles (Dugan and Randles, 1968; Dugan, 1972). Studies have been conducted which show that acidophilic *Thiobacilli* can be inhibited under laboratory situations (Dugan and Lundgren, 1964) but the practicality of inhibition in coal refuse piles has, as yet, not been demonstrated.

It has been reported that the anionic surfactants, alkylbenzene sulfonate (ABS) and sodium lauryl sulfate (SLS), were very active inhibitors of *T. ferrooxidans* when cultivated in the laboratory (Dugan and Lundgren, 1964). Figure 3A shows that 5 ppm ABS (approximately $1.4 \times 10^{-5}\text{M}$) effectively inhibits iron oxidation by a *T. ferrooxidans* cell suspension containing 4×10^7 cells/ml. Similar data indicates (fig. 3B) that SLS is an effective inhibitor at 2 ppm (approximately $7 \times$

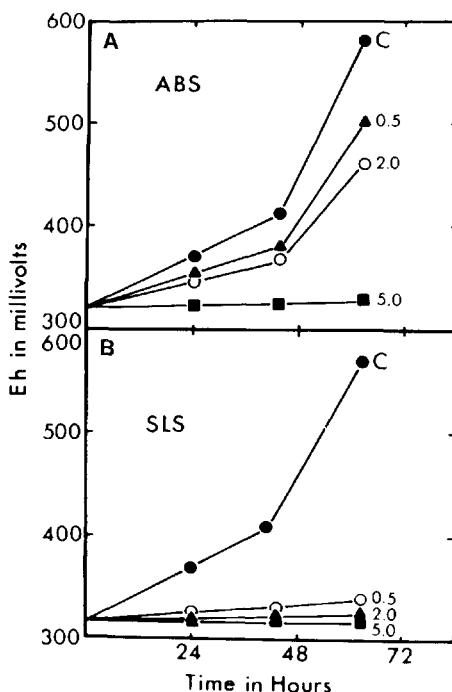


FIGURE 3. The effect of inhibitors on iron oxidation plotted as increase in Eh by *T. ferrooxidans* vs. time at $25 \pm 2^\circ\text{C}$. (A) alkylbenzene sulfonate (ABS) added at concentrations of 0.5, 2.0 and 5.0 mg/liter of culture medium. C is control with no ABS added. (B) sodium lauryl sulfate (SLS) added at concentrations of 0.5, 2.0 and 5.0 mg/l of culture medium.

10^{-6}M). The effectiveness of ABS was dependent upon the number of bacteria in suspension, as indicated in figures 4A and 4B where the number of cells in suspension was doubled (2x) and quadrupled (4x). SLS was not examined in this manner. Non-ionic detergents and some sulfonated organic compounds were much less toxic to growth than either ABS or SLS. It was also shown previously by Dugan and Lundgren that increase in the hydrogen potential (Eh, a measure of the oxidation/reduction potential) in this system was correlated to iron oxidation and to growth of the organism.

Subsequent laboratory investigations demonstrated that several different low molecular weight organic acids inhibited iron and sulfur oxidation as well as growth

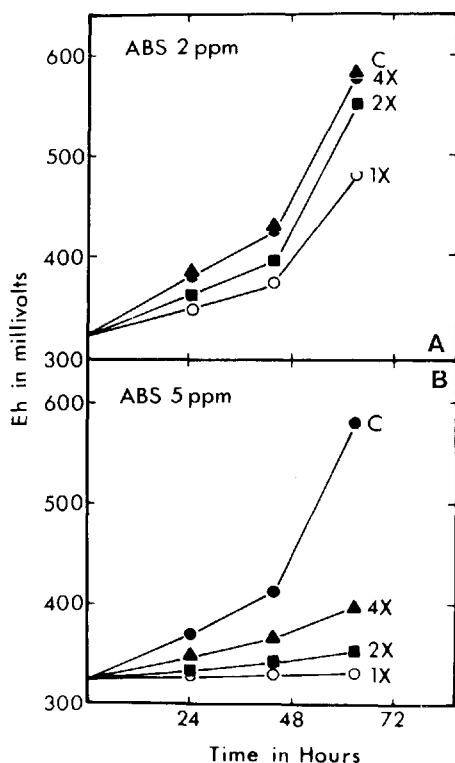


FIGURE 4. The effect of alkylbenzene sulfonate (ABS) on iron oxidation (Eh) by various cell densities of *T. ferrooxidans* vs. time at $25 \pm 2^\circ\text{C}$; (A) in the presence of 2.0 mg ABS/l and (B) 5.0 mg ABS/l. C=control with no added ABS. $1x = 4 \times 10^7$ cells/ml, $2x = 8 \times 10^7$ cells/ml, $4x = 1.6 \times 10^8$ cells/ml.

of *T. ferrooxidans*. When a suspension of *T. ferrooxidans* (8 mg cell wet weight per 5 ml of pH 3.0 H_2SO_4 which contained $5000 \mu\text{g Fe}^{2+}$ per ml as $\text{FeSO}_4 \times 7\text{H}_2\text{O}$) was aerated in the presence of added organic acids at 35°C ; the percentage of inhibition as compared to a control, which did not contain organic additives, was determined after 10 minutes. Figure 5 shows the effectiveness of formic, acetic and oxalic acids as inhibitors of iron oxidation by the bacteria and figures 6 and 7 show similar data for fumaric, succinic, malic, pyruvic and lactic acids.

Experiments similar to those described above (except that microbial iron oxidation was determined by addition of 5.0 ml of 2N HCl after specified time intervals)

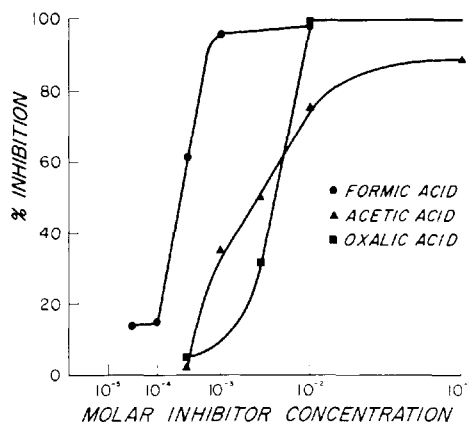


FIGURE 5. Percent inhibition of iron oxidation by washed *T. ferrooxidans* (8 mg cell wet wt/5 ml of solution containing 5 mg Fe^{++} per ml of pH 3.0 H_2SO_4) in the presence of various concentrations of formic, acetic, or oxalic acids after 10 min. at 35°C . Values are compared to controls in absence of organic acids defined as 0% inhibition.

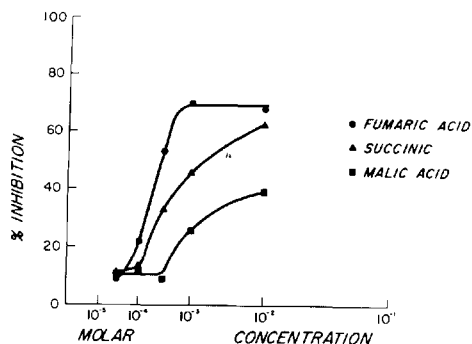


FIGURE 6. Inhibition of iron oxidation by *T. ferrooxidans* due to fumaric, succinic or malic acids. (Experimental data, similar to that shown in fig. 5).

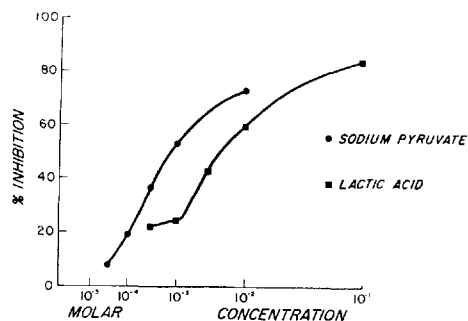


FIGURE 7. Inhibition of iron oxidation by pyruvic and lactic acids. (Data similar to that presented in figs. 5 and 6).

provided information on the rate of iron oxidation in the presence of organic acids. Figures 8 and 9 illustrate the effects of hexanoic and oxalacetic acids, respectively, and show that the rate of iron oxidation is markedly retarded by these organic acids. In these experiments, iron oxidation was determined by measuring the absorbance of the $\text{Fe}^{+3} + \text{HCl}$ complex at 410 nm.

Other investigators have demonstrated the inhibitory effects of several organic acids on both *T. ferrooxidans* and *T.*

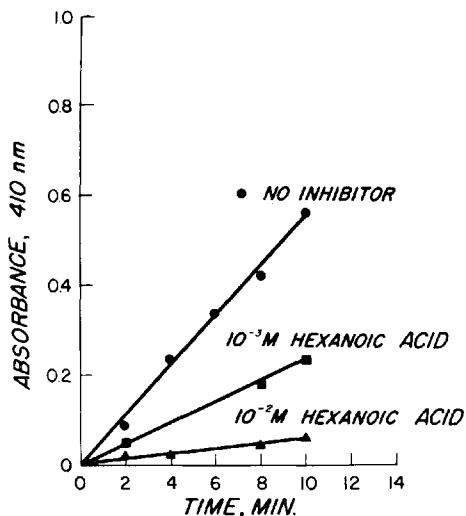


FIGURE 8. Curves showing the decrease in absorbance at 410 nm due to production of Fe^{+3} by *T. ferrooxidans* in the presence of 10^{-2}M or 10^{-3}M hexanoic acid vs. time at 35°C .

thiooxidans (Borichewski, 1967) and have also shown that organic acids are produced by these bacteria (Schnaitman and Lundgren, 1965). One practical consideration which should be mentioned is that various types of sewage sludge contain high percentages of volatile solids which have a significant content of organic acids. Addition of sludge to spoil banks would therefore tend to be inhibitory to the iron oxidizing bacteria and of course would add an organic or humic content to the spoils. Caution must be exercised, however, relative to the presence of viruses, pathogenic microorganisms and toxic minerals which may be present in certain sludges.

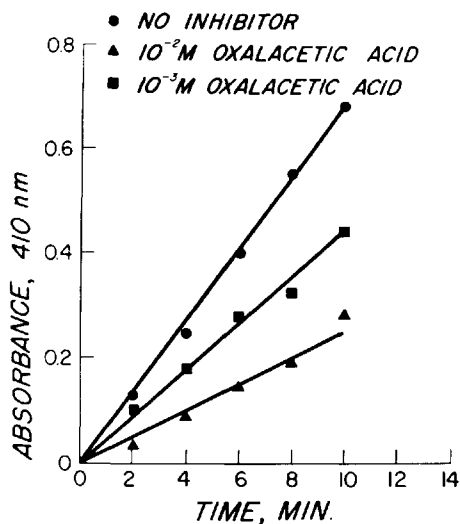


FIGURE 9. Decrease in absorbance at 410 nm in presence of 10^{-2}M or 10^{-3}M oxalacetic acid. (Data similar to that presented in fig. 8).

BIOLOGICAL EFFECTS OF ACID DRAINAGE

Mine drainage has a deleterious influence on fish, wildlife and plant life in receiving water (Boccardy and Spaulding, 1968; Riley, 1965; Temple and Kohler, 1954). Acid drainage also has caused a marked reduction in the microflora of non-acid streams and is particularly noxious to most aerobic and anaerobic heterotrophic bacteria that are indigenous to non-acid streams. Acid streams contain relatively low numbers of acid tolerant aerobic heterotrophic microbes which appear to originate in non-acid effluents such as field runoff. The acid tolerant heterotrophs increase in numbers to a maximum of about $2 \times 10^3/\text{ml}$ in acidic streams, until the pH decreases to approximately 3.0. These organisms then represent the heterotrophic aerobic microflora of streams which are comprised of a mixture of acid drainage and non-acid water. A stream composed entirely of acid drainage does not have a similar microflora. Most Gram positive aerobic and anaerobic heterotrophic bacteria die out very rapidly in acidic water and comprise a very small percentage of the total microbial population. Iron and sulfur oxidizing autotrophic bacteria are

always present in mine water and in streams contaminated by mine water (Tuttle *et al.*, 1968).

The toxic influence on anaerobic growth may be due to the high hydrogen potential (Eh) of the acid water which results from high concentrations of oxidized ions in solution. The adverse influence of acid drainage on the microflora of non-acid water may be due, in part, to removal of organic nutrients that are essential for heterotrophic growth via acid precipitation (Dugan and Randles, 1968; Tuttle *et al.*, 1968; 1969a). If the trophic base of the ecosystem is depleted, the higher trophic levels will also be proportionately depleted.

An attempt was made to assess the relative influence of three of the prominent chemical parameters that constitute acid mine drainage (i.e. H^+ , Fe^{3+} , SO_4^{2-}), as they effect the aerobic heterotrophic microflora of receiving streams. The approach was based on the premise that physiological activity of microorganisms is fundamental in triggering the ecological succession which ultimately results in re-establishment of higher forms of life (e.g. insects, fish, etc.) in the streams. Although iron, sulfate and hydrogen ions are all characteristic of acidic mine effluents, they appear to vary independently with respect to concentration in drainage which originates at different locations. It therefore becomes important to determine which component has the most deleterious influence on the biota; at what concentration the ion is deleterious; and whether or not significant interactions exist among ions as they influence biological processes. These con-

siderations have practical significance in that they could help to determine levels of abatement that are essential to allow recovery of acid polluted streams via natural processes. It was also desirable to know if a recognizable point exists at which growth of a normal aquatic microflora is inhibited and/or killed. This would allow prediction of the relative amount of acid pollution a stream could tolerate in a variety of circumstances and also to what extent the water would have to be treated. Three aerobic heterotrophic bacteria were isolated and selected for study from a non-acid stream (pH 6.2 to 8.2) which is located in the immediate vicinity of acid contaminated streams in Southeast Ohio (*Pseudomonas* M-1, *Flavobacterium* M-2, *Bacillus* M-3). A fourth, previously isolated, bacterium: *Pseudomonas* 229, was also studied.

Although the experimental details are reported elsewhere (McCoy and Dugan, 1968), the influence of the three chemical variables: pH in the range 2 to 7, ferric ion in the concentration range 1 to 100 $\mu g/ml$, and sulfate ion in the concentration range 50 to 5000 $\mu g/ml$ on survival of each of the bacteria was evaluated with the aid of computer analysis. Table 1 shows the mathematical models developed which represent the best fit of the four experimental variables. *Bacillus* M-3 was not included because sufficient data could not be derived from the test system for programming. Values which can be predicted on the basis of the models would be expected to vary from the experimental values by less than 0.2 log unit. Predictions were made on the basis of the models within the frame-

TABLE 1
Models representing the best fit of the data for three different bacteria.

PSEUDOMONAS	M1
LOG # BACT. = $32 - 21(pH) + 4.4(pH)^2 - 0.26(pH)^3 + 0.54 \text{ LOG}(Fe^{3+}) - 0.014(pH)^2 \text{ LOG}(SO_4^{2-})$	
PSEUDOMONAS	229
LOG # BACT. = $8.2 - 5.1(pH) + 1.3(pH)^2 - 0.074(pH)^3 - 0.64 \text{ LOG}(SO_4^{2-}) + 0.21 \text{ LOG}(Fe^{3+}) - (pH) \text{ LOG}(SO_4^{2-})$	
FLAVOBACTERIUM	M2
LOG # BACT. = $6.4 - 5.5(pH) + 1.4(pH)^2 - 0.084(pH)^3 + 0.12 \text{ LOG}(Fe^{3+})$	

work of our stated assumptions. Undoubtedly we have not considered all significant parameters involved in acidic streams. It was concluded that the test organisms should be able to grow in an acid stream when the pH is above 5.3, if iron levels are in the range 1–100 $\mu\text{g}/\text{ml}$ and sulfate levels are in the range 50–500 $\mu\text{g}/\text{ml}$.

Growth of the bacteria increased with increasing iron content provided the pH was 5 or above. Therefore we probably should not be overly concerned with the iron content of waters from a biological viewpoint until the concentrations are considerably in excess of 100 $\mu\text{g}/\text{ml}$. This comment does not relate to considerations such as corrosion and other non-biological situations. In general, there are complex relationships between sulfate and pH. That is, sulfate influences growth of the organisms but not in a linear manner. The variable of paramount importance appears to be H^+ , with some concern for sulfate.

One might anticipate response to our generalizations by biologists who are interested in the welfare of organisms

other than bacteria in streams. Microorganisms, once growing, will excrete metabolic byproducts into the environs which have buffering capacity. They also serve as nutrients for other organisms. The net result over a period of time is to re-establish a biological equilibrium which then includes higher life forms. It should also be emphasized that this study pertains only to effects of acid water on the heterotrophic microflora of "normal" streams. Our laboratory also has reported that a high population of heterotrophic aerobes are *indigenous* to certain highly acid (pH 2.5) water (Dugan *et al.*, 1970a; 1970b). This population of microorganisms represents an ecosystem entirely different from that found in the non-acid streams described above.

MICROORGANISMS INDIGENOUS TO ACID MINE WATER

An indigenous population of acid tolerant heterotrophic bacteria is responsible for production of slime streamers (figs. 10 and 11) in highly acid mine water (pH 2.8). This group of bacteria has not

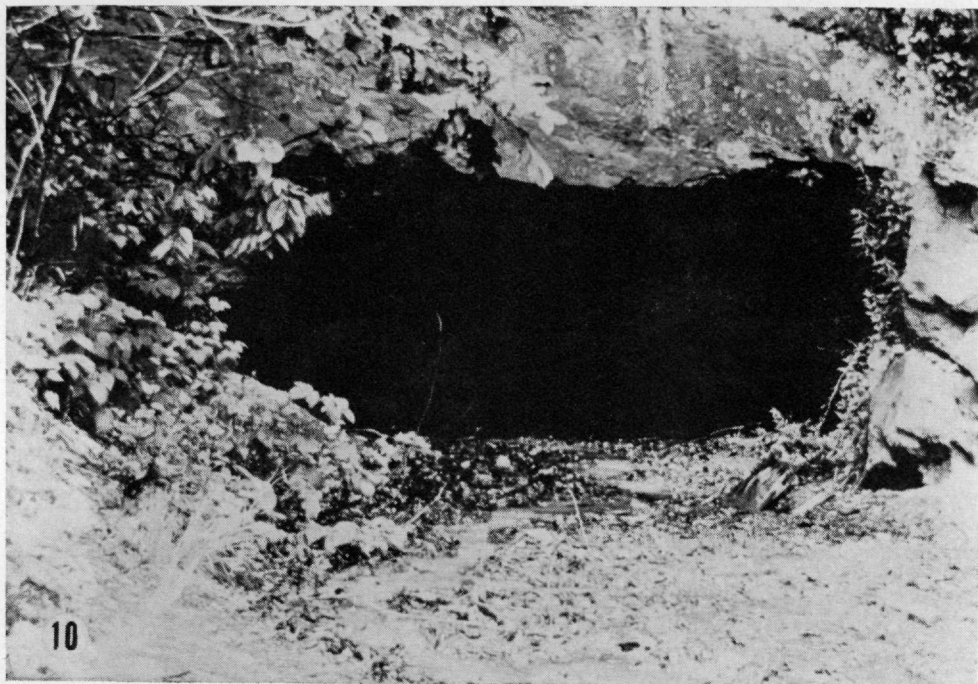


FIGURE 10. Photograph of the opening of an abandoned drift mine with acid drainage at pH 2.5. Height of mine opening is approximately 3 feet.

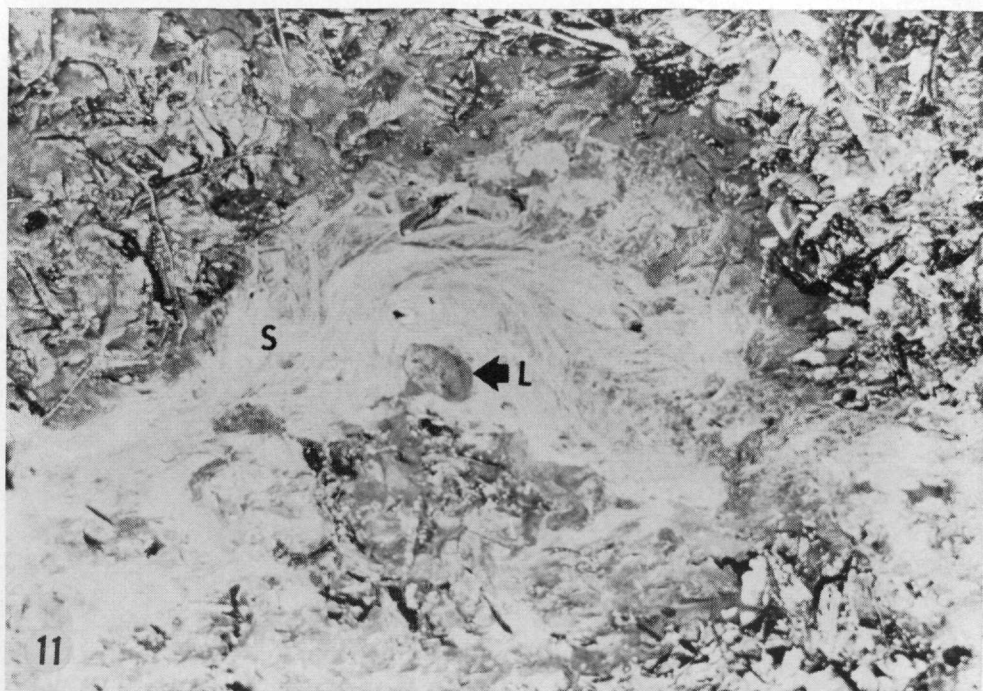


FIGURE 11. Photograph of "acid streamers" (S) due to bacterial growth in the drainage shown in fig. 10. A 3 inch leaf (L) in the water serves as a size comparison.

been well characterized but at least one isolate has a pH optimum near neutrality (Dugan *et al.*, 1970a; 1970b). This suggests that the organisms colonize in the stream creating a localized microcosm that is different from the surrounding, highly acid environment. An electron micrograph of a frozen etched preparation of the indigenous slime (fig. 12) shows bacteria embedded within a fibrous matrix which is comprised of extracellular polymer fibrils synthesized by the organisms. There appear to be inorganic crystals precipitated within the fibrous matrix.

Bohohloul and Brock (1974) have reported the growth of a thermophilic mycoplasma, *Thermoplasma acidophilum*, in refuse piles. Yeasts and filamentous fungi have frequently been isolated from acidic mine water (Cook, 1966; Dugan, 1968; Weaver, 1968). The source of organic nutrients required to support the rather extensive amount of heterotrophic growth in mine drainage is not known. One possible source would be from metabolic by-products produced by acido-

philic autotrophs. Schnaitman and Lundgren (1965) reported that organic acids accumulate from autotrophic growth of *T. ferrooxidans*. Several species of algae have also been observed in rather large numbers in acid mine drainage in the presence of sunlight, especially *Euglena* and *Ulothrix* (Dugan, 1968; Joseph, 1953; Lackey, 1938), which may also enrich the stream with organic substances. Other sources of heterotrophic nutrient would be coal and the organic content of other sedimentary deposits. We also have observed the blood worm, *Chironomus*, living in the slime streamers in acid drainage.

Growth of such a variety of microorganisms in sulfuric acid environments down to pH 2.0 leads to the conclusion that our concept of an extreme environment is only relative to that with which we are familiar and that this environment is not extreme to many microorganisms. Conditions are present which will promote recovery of acid contaminated streams to what we consider "normal" if the acid pollution is terminated.

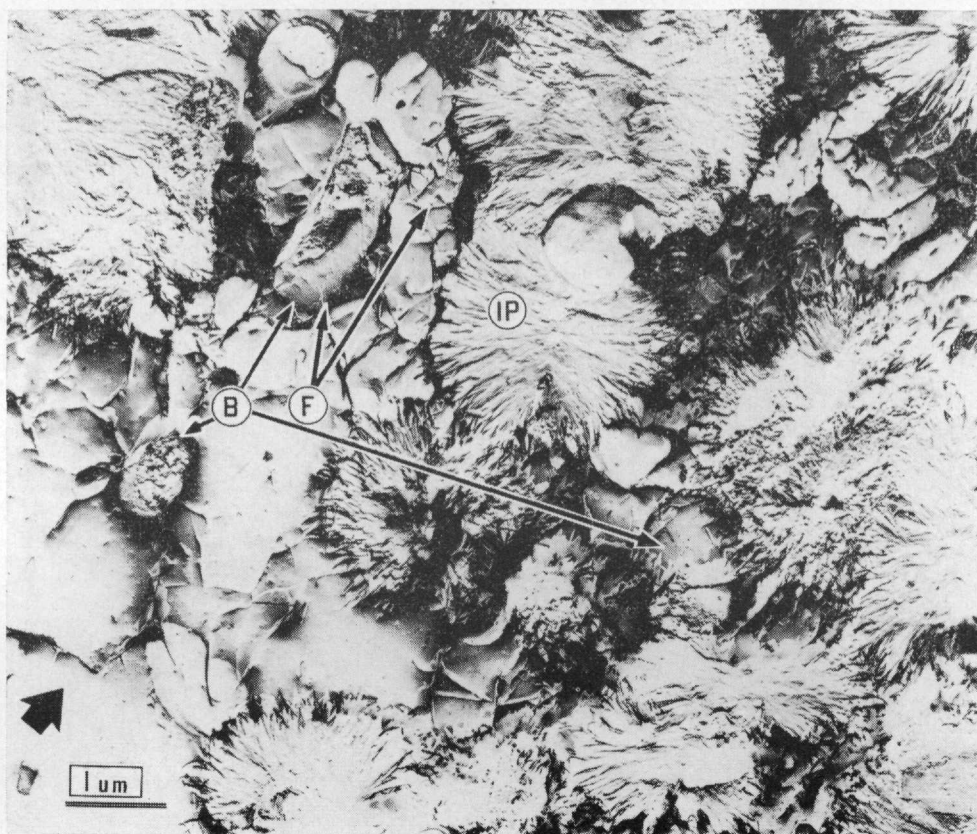


FIGURE 12. Electron micrograph of a frozen etched preparation of the acid slime shown in fig. 11. Bacteria (B) embedded within a matrix of fibrillar strands (F) synthesized by the organisms can be observed. What appear to be crystals of an inorganic precipitate (IP) embedded within the slime fibrils are also present.

BACTERIAL REDUCTION OF SULFATE TO SULFIDE

Dissimilatory sulfate reducing bacteria are also capable of growth in acidic mine drainage; provided that sufficient organic material is present to promote consumption of oxygen and reduce the Eh of the system. These bacteria are obligate anaerobes and belong to two genera, *Desulfovibrio* and *Desulfotomaculum*. They are not acidophilic but are somewhat acid tolerant. Apparently organic and other microparticulates allow a localized microcosm to develop around the cells which has a pH higher than that of the bulk solution. Growth of both genera is accompanied by an increase in pH and reduction of $\text{SO}_4^{=}$ to $\text{S}^=$, which is precipitated as black FeS in mine water. The

potential utility for microbial sulfate reduction as an acid mine water abatement procedure has been described elsewhere (Dugan, 1972, Tuttle *et al.*, 1969a; 1969b). Organic nutrients for this group of bacteria can be supplied from composting leaves and other natural detritus, sawdust, algal growth, sewage sludge, etc. Some caution relative to the use of sewage sludge on refuse piles is indicated, because the high organic content accompanied by high sulfate concentrations can result in anaerobic conditions and selection of the dissimilatory sulfate reducing bacteria. This would result in production of hydrogen sulfide (H_2S —a highly toxic gas with an extremely objectionable odor) which may or may not be precipitated as metallic sulfide (e.g. FeS) depending upon the concentration of

metal ion present relative to the sulfate concentration (Dugan, 1972).

Acknowledgments.—Portions of this work were supported by: Grant No. GB5955 from the National Science Foundation, Grant No. 14-01-0001-805, 980 from the U.S. Office of Water Resources Research. Data reported in figures 5 through 9 were part of the Ph.D. Dissertation of Dr. Jon Tuttle, Ohio State Univ. 1969. The frozen etched specimen (fig. 12) was prepared by Dr. C. H. Remsen.

LITERATURE CITED

- Acid Mine Drainage in Appalachia. 1969. Appendix C: The incidence and formation of mine drainage pollution in Appalachia. 253 pp. Appendix F: Impact of mine drainage on stream ecology. 65 pp. U.S. Corps of Engineers and U.S. Dept. of Interior.
- Beck, J. 1960. A ferrous-ion-oxidizing bacterium. I. Isolation and some general physiological characteristics. *J. Bacteriol.* 79: 502-509.
- Belly, R. T. and T. D. Brock. 1974. Ecology of iron-oxidizing bacteria in pyritic materials associated with coal. *J. Bacteriol.* 117: 726-732.
- Blaylock, B. A. and A. Nason. 1963. Electron transport systems of the chemoautotroph *Ferrobacillus ferrooxidans*. I. Cytochrome c-containing iron oxidase. *J. Biol. Chem.* 238: 3453-3462.
- Boccardy, J. A. and W. M. Spaulding. 1968. Effects of surface mining on fish and wildlife in Appalachia. Report U.S. Dept. Interior, Bureau of Sports, Fisheries and Wildlife.
- Bodo, C. A. and D. G. Lundgren. 1974. Iron oxidation by cell envelopes of *Thiobacillus ferrooxidans*. *Can. J. Microbiol.* 20: 1647-1652.
- Bohoolool, B. B. and T. D. Brock. 1974. Immunofluorescence approach to the study of the ecology of *Thermoplasma acidophilum* in coal refuse material. *Applied Microbiol.* 28: 11-16.
- Borichewski, R. M. 1967. Keto acids as growth-limiting factors in autotrophic growth of *Thiobacillus thiooxidans*. *J. Bacteriol.* 93: 597-599.
- Carpenter, E. V. and L. K. Henderson. 1933. Acid mine drainage from bituminous coal mines. Res. Bull. No. 10. Engineering Experiment Station, Univ. of West Virginia.
- Colmer, A. R. and M. E. Hinkle. 1947. The role of microorganisms in acid mine drainage: A preliminary report. *Science* 106: 253-256.
- Colmer, A. R., K. L. Temple and M. E. Hinkle. 1949. An iron oxidizing bacterium from the acid drainage of some bituminous coal mines. *J. Bacteriol.* 59: 317-328.
- Cook, W. B. 1966. The occurrence of fungi in acid mine drainage. *Proc. Indust. Waste Conf.* 21: 258-274.
- Dewey, D. L. and J. Beecher. 1966. The internal hydrogen ion concentration of *Thiobacillus thiooxidans* and survival after irradiation. *Radiation Res.* 28: 289-295.
- Din, G. A., I. Suzuki and H. Lees. 1967. Ferrous iron oxidation by *Ferrobacillus ferrooxidans*. Purification and properties of Fe^{++} -cytochrome C reductase. *Can. J. Biochem.* 45: 1523-1546.
- Dugan, P. R. and D. G. Lundgren. 1964. Acid production by *Ferrobacillus ferrooxidans* and its relation to water pollution. *Develop. in Industr. Microbiol.* 5: 250-257.
- and C. I. Randles. 1968. The microbial flora of acid mine water and its relationship to formation and removal of acid. Rept. Ohio State Univ. Water Resources Center, Columbus, Ohio. 123 pp.
- C. B. Macmillan and R. M. Pfister. 1970a. Aerobic heterotrophic bacteria indigenous to pH 2.8 acid mine water: Microscopic examination of acid streamers. *J. Bacteriol.* 101: 973-981.
- 1970b. Aerobic heterotrophic bacteria indigenous to pH 2.8 mine water: Predominant slime-producing bacteria in acid streamers. *J. Bacteriol.* 101: 982-988.
- 1972. Biochemistry of acid mine drainage. In *Biochemical Ecology of Water Pollution*. pp. 123-137. Plenum Publ. Co., N.Y.
- J. Landesman and C. C. Walden. 1967. Role of *Thiobacillus ferrooxidans* in the oxidation of sulfide minerals. *Can. J. Microbiol.* 13: 397-403.
- Good, D. M., V. T. Ricca and K. S. Shumate. 1970. The relation of refuse pile hydrology to acid production. In 3rd Symp. on Coal Mine Drainage Research, Mellon Inst., Pittsburgh. pp. 145-151.
- Howard, A. and D. Lundgren. 1970. Inorganic pyrophosphatase from *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). *Can. J. Biochem.* 48: 1302.
- Joseph, J. M. 1953. Microbiological study of acid mine waters: Preliminary Report. *Ohio J. Sci.* 53: 123-137.
- Lackey, J. B. 1938. The flora and fauna of surface waters polluted by acid mine drainage. *Public Health Rep.* 53: 1499-1507.
- Land Reborn. 1974. Report by: Board on on Unreclaimed Strip Mined Lands and Ohio Department of Natural Resources. Columbus, Ohio. 91 pp.
- Lau, C. M., K. S. Shumate and E. E. Smith. 1970. The role of bacteria in pyrite oxidation kinetics. *Proc. 3rd Symp. Coal Mine Drainage*. Mellon Inst., Pittsburgh. pp. 114-122.
- Leathan, W. W., S. A. Braley and L. D. McIntyre. 1953. The role of bacteria in the formation of acid from certain sulfuritic constituents associated with bituminous coal. I. *Thiobacillus thiooxidans*. *Appl. Microbiol.* 1: 61-64. II. Ferrous iron oxidizing bacteria. *Appl. Microbiol.* 1: 65-68.
- Lees, H., S. C. Kwok and I. Suzuki. 1969. The thermodynamics of iron oxidation by the ferrobacilli. *Can. J. Microbiol.* 15: 43-46.
- Liptak, P. 1974. Restoration of strip mining sites. In *Environ. Engineers Handbook Vol. 1.* (Ed.) B. G. Liptak. pp. 1822-1833.

- Lorenz, W. C. and R. W. Stephan. 1967. Factors that affect the formation of coal mine drainage pollution in Appalachia. Rept. Bureau of Mines, U.S. Dept. Interior. Pittsburgh.
- McCoy, B. and P. R. Dugan. 1968. The activity of microorganisms in acid mine water. II. The relative influence of iron, sulfate and hydrogen ions on the microflora of a non-acid stream. In 2nd Symp. Coal Mine Drainage Research. Mellon Inst., Pittsburgh. pp. 64-79.
- Osborn, E. F. 1974. Coal and the present energy situation. *Science* 183: 477-481.
- Riley, C. V. 1965. Limnology of acid mine water impoundments. 1st Symp. Acid Mine Drainage Research. Mellon Inst., Pittsburgh.
- Schnaitman, D. and D. G. Lundgren. 1965. Organic compounds in the spent medium of *Ferrobacillus ferrooxidans*. *Can. J. Microbiol.* 11: 23-27.
- Shumate, K. S., E. E. Smith, P. R. Dugan, R. A. Brandt and C. I. Randles. 1971. Acid Mine Drainage Formation and Abatement. Report U.S. Environ. Protect. Agency, Water Pollution Control DAST-42, 14010 FPR. Washington, D.C.
- Silver, M. and D. G. Lundgren. 1968a. Sulfur oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). *Can. J. Biochem.* 46: 457-461.
- . 1968b. The thiosulfate oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). *Can. J. Biochem.* 46: 1215-1220.
- Silverman, M. P. and H. L. Ehrlich. 1964. Microbial formation and degradation of minerals. *Adv. in Appl. Microbiol.* 4: 153-206.
- Silverman, M. P. 1967. Mechanism of bacterial pyrite oxidation. *J. Bacteriol.* 94: 1046-1051.
- Singer, P. C. and W. Stumm. 1970. Acidic mine drainage: The rate limiting step. *Science* 167: 1121-1124.
- Temple, K. L. and W. A. Kohler. 1954. Drainage from bituminous coal mines. Res. Bull. No. 25, Engineering Exp. Station, Univ. of West Virginia, Morgantown.
- Truax-Traer Coal Co. 1971. Control of mine drainage from coal mine mineral wastes. Phase I. Hydrology and Related Experiments. Reports. U.S. Environ. Protection Agency, Water Pollution Control. Res. 14010DDH. 148 pp.
- Tuttle, J. H., C. I. Randles and P. R. Dugan. 1968. Activity of microorganisms in acid mine water. I. Influence of acid water on aerobic heterotrophs of a normal stream. *J. Bacteriology* 95: 1495-1503.
- Tuttle, J. H., P. R. Dugan, C. B. Macmillan and C. I. Randles. 1969a. Microbial dissimilatory sulfur cycle in acid mine water. *J. Bacteriol.* 97: 594-602.
- Tuttle, J. H., P. R. Dugan and C. I. Randles. 1969b. Microbial sulfate reduction and its potential utility as a water pollution abatement procedure. *Appl. Microbiol.* 17: 297-302.
- Vestal, R. and D. G. Lundgren. 1971. The sulfate oxidase of *Thiobacillus ferrooxidans*. (*Ferrobacillus ferrooxidans*). *J. Bacteriol.* 95: 1851-1856.
- Vimmerstedt, J. P., J. H. Finney and P. Sutton. 1973. Effect of Strip Mining on Water Quality. Report Ohio State Univ. Water Resources Center to Office of Water Resources Research, U.S. Dept. Interior. 54 pp.
- Vogler, K. G. and W. W. Umbreit. 1941. The necessity for direct contact in sulfur oxidation by *Thiobacillus thiooxidans*. *Soil Sci.* 51: 331-337.
- Walsh, F. and R. Mitchell. 1972. A pH-dependent succession of iron bacteria. *Environ. Sci. Technol.* 6: 809-812.
- Weaver, R. H. and H. D. Nash. 1968. Effects of strip mining on the microbiology of a stream free from domestic pollution. In 2nd Symp. Coal Mine Drainage. Mellon Inst., Pittsburgh. pp. 80-97.